IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Uri GALILI *et al.*
Title:	IMMUNE TOLERANCE TO PREDETERMINED ANTIGENS
Appl. No.:	10/789,955
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Art Unit:	1644
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DECLARATION UNDER 37 C.F.R. § 1.132 OF URI GALILI

I, Uri Galili, state and declare that:

1. I am a citizen of U.S.A residing at: 27/5 Eaglehead Terrace, Shrewsbury, MA 01545.

2. I am a PhD working as a Professor in the Department of Medicine at University of Massachusetts School of Medicine, Worcester, MA 01006.

3. I have a Ph.D. in Immunology and Experimental Medicine.

4. I am a coinventor of U.S. Application Serial No. 10/789,955, filed February 27, 2006 ("the application").

5. I, and/my co-inventor, Haruko Ogawa, performed or caused to be performed the following experiments relating to the induction of immune tolerance in a mammal. The methods used in this experiment were performed as described in the application, and results were obtained through routine experimentation. Any differences between the application methods and the experimental methods described herein are solely the result of conventional methods, well-known to one of skill in the art (*e.g.*, methods of introducing vectors into lymphocyte cells, methods of testing for the presences of antibody or antigen, etc.).

6. Spleen lymphocytes were isolated from Balb/c mice, and suspended in the Mouse T cell Nucleofection Medium (Amaxa GmbH, Cologne, Germany) at 10-20×10^6 lymphocytes in 100 µl. Two expression vectors, pcDNA3 (Invitrogen, Carlsbad, CA) containing human H transferase (HT) and/or B transferase (BT) gene, were added to the cell as 1-2 µg in 100 µl. The cell suspension was transferred into a cuvette, and applied to the Nucleofector (Amaxa) according to the manufacturer's instruction.

7. Following the nucleofection, the lymphocytes were *in vitro* cultured for 4 hours, then washed and intravenously administered into syngeneic mice as 5-10×10^6 lymphocytes per mouse. Control mice received the lymphocytes nucleofected with mock vector. The procedure above was repeated on the same recipient mice for 4 times in 3-4 day intervals.

8. Subsequently, the mice were intraperitoneally immunized with 10 mg of human blood type B red cell membranes (HBM) for 4 times with 2 weeks interval.

9. Two weeks after the last immunization, sera were collected from the mice and the production of anti-B antibodies in the mice was analyzed by ELISA using synthetic blood group B trisaccharide conjugated with bovine serum albumin (Dextra, Reading, UK) as solid phase antigen, and HRP-conjugated anti-mouse IgG (Cappel, Autrora, OH) or IgM (Rockland, Gilbertsville, PA). The color developed with TMB substrate reagent (BD Biosciences, San Diego, CA) was measured at OD_{450} nm.

10. Separately, the nucleofected lymphocytes were *in vitro* cultured for 14 hours and the expression of B antigen on the cells was analyzed by flow cytometry using monoclonal antibody against human blood group B antigen (Dako Cytomation, Carpinteria, CA).

11. Approximately 10% of mouse lymphocytes expressed blood group B Ag 14 hours after the nucleofection with both of HT and BT genes since blood group B is synthesized on blood group H. Such an expression was not observed by nucleofection of the BT gene alone. Control mice developed anti-B IgG and IgM following the HBM immunizations. In contrast, the mice that received nucleofected lymphocytes expressing B antigen did not develop anti-B antibodies, suggesting that those mice lacked anti-B immune response.

12. Results demonstrating that tolerance was induced to naïve anti-B B cells by nucleofected lymphocytes is shown in the figures presented in Appendix A. Figure (a) shows the production of anti-B IgG in control mice and tolerized mice. Both groups had been immunized four times with HBM after receiving either lymphocytes containing a mock vector (control mice) or lymphocytes containing the HT and BT vectors. The anti-B response in 6 mice that received lymphocytes nucleofected with HT and BT vectors is represented by the closed circle (●); the anti-B response in 5 control mice that received lymphocytes containing the mock vector is represented by the open circle (○). Figure (b) shows the production of anti-B IgM in the control and tolerized mice immunized four times with HBM. Data are presented as in (a). Statistical analysis by Student's t-test indicated significant differences between the two groups; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

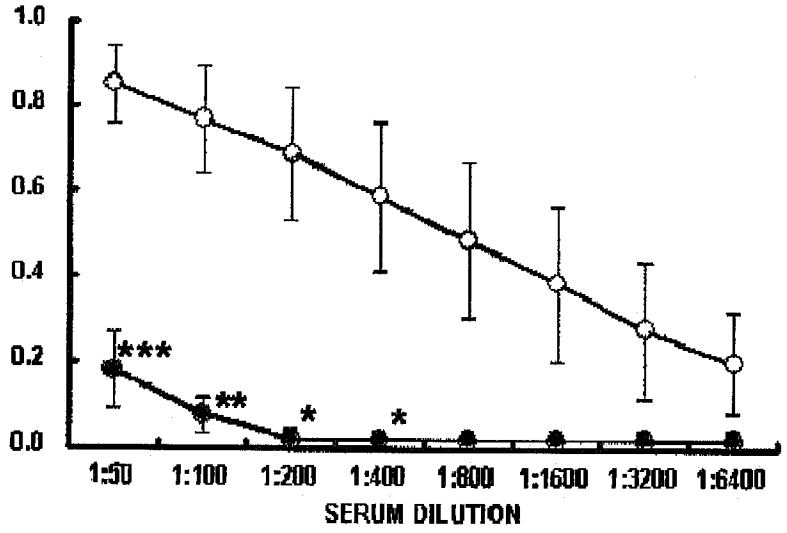
13. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or of any patent resulting therefrom.

Date: November 17, 2006 By: Uri Galili

Uri Galili

APPENDIX A

(a)



(b)